- Search History will be lost after eight hours of inactivity.
- To combine searches use # before search number, e.g., #2 AND #6.
- Search numbers may not be continuous; all searches are represented.
- Click on query # to add to strategy

Search	Most Recent Queries	Time	Result
#13	Search **degli-esposti**	16:30:14	308
#11	Search **#8 and soluble proteins**	09:07:51	8
#10	Search **#8 and tnf**	09:07:43	0
#9	Search **#8 and mammalian cells**	09:07:27	7
#8	**Related Articles** for **PubMed** (Select **14701904**)	09:07:08	169
#6	Search **#3 and mammalian cells**	09:06:58	1
#5	Search **#1 and mammalian**	09:06:50	0
#4	Search **#1 and mammalian cells**	09:06:45	0
#3	**Related Articles** for **PubMed** (Select **14703990**)	09:06:31	343
#1	Search **reduce protein aggregates and reduced temperature**	09:05:45	3

Clear History

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cules to the profound phenotypes accompanying the *lpr* mouse in the Fas case (13) and the hyper IgM globulinemia associated with CD40 ligand defects in humans (14). TNF, on the other hand, benefitted from its natural existence as a soluble mediator and the ready availability of a recombinant form. We have taken the approach of converting the surface LT heteromeric complexes into a TNF-like secreted form. The generation of the surface LT complexes in soluble forms has allowed us to examine several key facets of this unusual structure. First, the LT-α and LT-β stoichiometry in the soluble complexes could be unambiguously determined. Next we were able to assess the stability of the trimers and ascertain whether scrambling could occur. Such interconversions would vastly complicate any biological studies performed using the soluble molecules as mimics of the surface forms. The receptor binding properties of these ligands will be analyzed in a subsequent paper.[2]

MATERIALS AND METHODS

Baculovirus Constructs

The baculovirus constructs encoding for LT-α and a truncated version of LT-β with a 10-amino acid *myc* tag have been described (8). An altered form of LT-β that retained the extracellular domain and the VCAM-1 leader sequence yet lacked the *myc* tag was prepared by ligating the VCAM-1 signal sequence to a polymerase chain reaction product of LT-β. A *Not*I blunt end fragment of the signal sequence was obtained as described (8). The LT-β fragment was produced by polymerase chain reaction amplification off the cDNA with the primers 5′-GACCCCGGGGCACAGGCCCAG-3′ and 5′-CAGTGCGGCCGCTCACGCACTCGCACCAC-3′ using *Pfu* DNA polymerase (Stratagene, La Jolla, CA) in 20 mM Tris-HCl, pH 8.75, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.1 mg/ml bovine serum albumin, 10% Me_2SO, and 0.2 mM dNTPs for 25 cycles at 94 °C for 30 s, 63 °C for 1 min, and 72 °C for 2 min with a 5-min extension at 72 °C. The polymerase chain reaction product was gel purified, cut with *Not*I, and ligated to the VCAM-1 signal sequence and *Not*I-linearized BlueBacIII. The *Not*I mutant of BlueBacIII (Invitrogen, San Diego, CA) was a gift from E. Garber (Biogen). Recombinant virus were produced and purified as described previously (8).

To create a mutated version of LT-α in which Asp^{50} was replaced by Asn, a *Not*I fragment containing the entire human LT-α gene (5) was subcloned into a pUC8 derivative, pNN11. Unique site elimination mutagenesis (Pharmacia Biotech Inc.) was used to alter the Asp^{50} codon, GAC, to AAC (Asn). Plasmids containing the mutation were screened for the loss of a *Rsr*II site and then confirmed by DNA sequence analysis. The entire *Not*I fragment was sequenced to ensure that extraneous mutations were not introduced during the new strand synthesis reaction. For expression of the mutant protein in insect cells, the gel purified *Not*I fragment was ligated into *Not*I linearized, dephosphorylated BlueBacIII. Recombinant virus were produced and plaque purified as described by Invitrogen. For homo- or heterotrimer production, the LTα D50N virus was used to infect High Five® (Invitrogen) cells alone or in combination with the VCAM/c-*myc*-tagged wild type LT-β virus.

Preparation of Receptor Affinity Resins

LT-β-R (LTβ-R-Fc) and TNF-R55 (TNF-R55-Fc) immunoglobulin chimeras were constructed as described previously (15). Chinese hamster ovary cells expressing either LTβ-R-Fc or TNF-R55-Fc were grown in suspension for 10–14 days in a Dulbecco's modified Eagle's medium/Ham's F-12-based medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum, 10 μg/ml bovine insulin, 10 μg/ml bovine transferrin, and 0.1% F-68 shear protectant (Life Technologies, Inc.). At harvest, sodium azide was added to a final concentration of 0.05%, and the conditioned medium was clarified by sequential dead end filtration using a 5-micron followed by a 0.3-micron Polygard filter cartridges (Millipore, Bedford, MA). The filtrated medium was concentrated by ultrafiltration using a S10Y30 spiral cartridge system (Amicon, Danvers, MA) and stored at −20 °C. The receptor-Fc chimeras were purified by Protein A affinity chromatography (POROS A column, PerSeptive Biosystems, Framingham, MA). To prepare LT-β-R-Fc and TNF-R55-Fc affinity resins, both preparations were immobilized on cyanogen bromide-activated Sepharose 4B (Pharmacia) overnight in 0.1 M sodium borate, 0.5 M NaCl, pH 8.5, at a final concentration of 5 mg of receptor-Fc/ml of resin. After blocking any underivatized sites with 100 mM ethanolamine, pH 8.0, for 1 h, the receptor affinity resins were washed with 25 mM sodium phosphate, 100 mM NaCl, pH 2.8 (elution buffer) prior to use and stored in 20 mM sodium phosphate, 150 mM NaCl, pH 7.0 (PBS), supplemented with 0.02% sodium azide.

Generation of LT-α/β Containing Conditioned Medium from Baculovirus-infected Insect Cells

To prepare LT-α/β heteromers, High Five® (Invitrogen) insect cells were grown to late log phase in a bioreactor controlled for oxygen containing 10 liters of sf900II medium (Life Technologies, Inc.) at 28 °C. The cells were infected at a density of 2.2×10^6 cells/ml with two different recombinant baculovirus preparations coding for LT-α and LT-β at a multiplicity of infection of 2 and 7, respectively. Fresh sf900II medium (1.5 liters) was added at the time of infection, and the culture was harvested 49 h post infection. Cells and cell debris were removed by centrifugation. A protease inhibitor mixture containing phenylmethylsulfonyl fluoride (Sigma) and EDTA were added to the supernatant at a final concentration of 0.15 mM and 1 mM, respectively, prior to filtration using a 10-inch 5-micron followed by a 10-inch 0.3-micron spiral Polygard cartridge filter (Millipore). The clarified insect cell supernatant containing LT-α/β was concentrated 10-fold by ultrafiltration using three S1Y10 spiral membrane cartridges (Amicon) connected in series. The concentrate was stored frozen at −70 °C. To prepare LT-α3, sf9 insect cells (Life Technologies, Inc.) were grown in the bioreactor to late log phase in 9 liters sf900II culture medium. The cells were infected with baculovirus coding for LT-α at a multiplicity of infection of approximately 5. The culture was harvested 48 h post infection and processed as described for the LT-α/β co-infected culture.

Affinity Purification of LT Forms

To purify the LT-α/β heteromers contained in the insect cell conditioned medium, the concentrate was loaded onto a TNF-R55-Fc affinity column at a flow rate of 0.5 ml/min. The flow-through material was stored at 4 °C, and the column was sequentially washed with 5 column volumes of PBS, 5 column volumes of PBS supplemented with 500 mM NaCl, and again with 5 column volumes of PBS. The bound material was eluted with elution buffer into fractions containing a 5% final volume of 500 mM sodium phosphate, pH 8.6 (neutralization buffer). To ensure that all the free LT-α was removed, the flow-through material from the first TNF-R55-Fc column was reloaded onto a second TNF-R55-Fc column, and the flow-through was again collected. The elution fractions from the two TNF-R55-Fc column runs that contained LT-α3 and LT-α2/β1 were identified by UV absorbance and pooled. To obtain pure LT-α2/β1, the combined TNF-R55-Fc elution pools were immediately loaded onto a LT-β-R-Fc affinity column equilibrated in PBS at a flow rate of 0.5 ml/min. The column was washed and eluted as described for the TNF-R55-Fc affinity column. To obtain pure LT-α1/β2, the flow-through from the second TNF-R55-Fc column was loaded onto a LT-β-R-Fc affinity column at a flow rate of approximately 0.5 ml/min. The column was washed as described for the TNF-R55-Fc column and eluted with the elution buffer at pH 3.5 instead of pH 2.8. To obtain pure LT-α3 trimers, the concentrate from the insect cell culture infected with the LT-α virus only was purified using a TNF-R55-Fc affinity column. The bound proteins were washed as described earlier, and the column was eluted with elution buffer at pH 3.5 into tubes containing 5% neutralization buffer. For all affinity purifications, the fractions containing protein were identified by UV absorbance, pooled, and analyzed on 10–20% gradient SDS-PAGE gels (Daiichi, distributed by Integrated Separation Systems, MA). The purified LT preparations were stored in portions at −70 °C.

Purification of LT-α and LT-α/β Multimers Containing the D50N Mutated LT-α Form

Human D50N mutated LT-α1/β2 and LT-α2/β1 were purified using LT-β-R-Fc affinity followed by cation exchange chromatography. The two heterotrimers were isolated from clarified culture supernatant on a LT-β-R-Fc Sepharose column essentially as described for the wild type LT trimers and were eluted with elution buffer at pH 2.8 and immediately neutralized. To separate D50N mutated LT-α1/β2 from LT-α2/β1 contained in the LT-β-R-Fc Sepharose elution pool, the sample was diluted with two volumes of buffer A (see below) and chromatographed on a carboxymethyl column as described below under "Analytical Methods."

[2] J. L. Browning, manuscript in preparation.

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portion was removed with *Hin*dIII and placed into the *Hin*dIII site of pRcCMV or pRcRSV (Invitrogen) yielding CH202 or CH203, respectively. The CHO cells secreting hLT-α (clone 5) under methotrexate selection (17) were transfected by electroporation with 20 μg of *Msc*I-linearized pRcCMV LT-β or pRcRSV LT-β and 0.5 μg of *Aat*II-linearized pSV-Neo. The *Msc*I site in the pRcCMV or pRcRSV vectors bisects the neomycin resistance gene, rendering it dysfunctional. Neomycin-resistant cells were selected with 0.7 mg/ml G418, positive clones were analyzed by FACS, and subclones were isolated. To express LT-β alone in CHO cells, the *Hin*dIII cassette from CH202 was transferred into pMDR901 to form CH224. The vector was linearized with *Aat*II and transfected by electroporation into CHO dihydrofolate reductase-cells by using a high copy protocol. Cells resistant to 200 nM methotrexate were isolated and analyzed by FACS.

Cos cells were transfected by electroporation with the parent vector pCDM8, pCDM8-LT-β (clone 12) or the original pCDM8-LT-β with the ATG substitution (pCDM8-LT-β-Met) either with or without the pCDM8-LT-α clone described earlier (8). Cells were analyzed by FACS after 2.5 days.

Receptor-Fc chimeras

The p55 TNFR-Fc construct was prepared by PCR amplification of the extracellular domain from an activated II-23 cDNA library by using the primers: 5′ CCGGATCCGCGGCCGCCATGGGCCTCTCCACCGT G3′ and 5′GATTGTCGACTGTGGTGCCTGAGTCCTC3′. The amplified fragment was digested with *Not*I and *Sal*I, and ligated into *Not*I-linearized pSAB132 along with a *Sal*I/*Not*I fragment containing the Ig Fc domain as described (31), yielding the vector pSAB132-p55 TNFR-Fc. The LT-β-R-Fc construct was likewise prepared by PCR amplification of the extracellular domain by using the primers 5′ AACTGCAGCGGC CGCCATGCTCCTGCC3′ and 5′GACTTTGTCGACCATGGTTCCT GACATCTCTGG3′. Amplification was performed by using a cDNA clone encoding the entire LT-β-R obtained from P. Marynen (University of Leuven, Leuven, Belgium) (32). The amplified product was digested with *Not*I and *Sal*I and ligated as described above, yielding the vector pSAB132-LT-β-R-Fc. Protein from each construct was obtained as supernatants from transiently transfected Cos cells containing greater than 0.5 μg/ml. Additionally, the *Not*I insert from each construct was transferred into pMDR901 and used to isolate stable CHO cell lines expressing receptor-Fc chimeras by using methotrexate selection. Pure protein was purified from either source by protein A Sepharose affinity chromatography.

Analytical methods

FACS analyses were performed basically as described earlier by using a FACScan instrument (11) with 10 μg/ml phycoerythrin-labeled goat anti-human IgG or goat anti-mouse IgG (Calbiochem, San Diego, CA) as second Abs. Staining experiments with the mouse mAbs were conducted on ice in RPMI 1788 with 10% FCS and 50 μg/ml human IgG (heat aggregated in some cases). Receptor-Fc staining was performed on ice in the same RPMI solution, however, the human IgG was substituted with 5% heat-aggregated normal rabbit serum. In all cases with human tumor lines, cell supernatants from transiently transfected Cos cells were compared with material purified from stable CHO lines by using protein A affinity chromatography to control for receptor aggregation occurring during purification. Receptor aggregation is problematic with the purified LT-β-R-Fc construct, but has not been observed with the crude supernatants. In further experiments, anti-FcRII- and anti-FcRIII-blocking mAbs, IV-3 and 3G8 (Mederex, West Lebanon, NH) were used to exclude FcR background contributions. The aggregated form can bind an FcR receptor even in the presence of the above blocking mAbs and this signal is presumably mediated via an FcRII isoform that is not blocked by the IV-3 mAb. Careful comparison of crude LT-β-R-Fc containing supernatants vs the purified LT-β-R-Fc allowed confirmation of specific binding in all the experiments described in this study.

Radiolabeling either biosynthetically or via surface iodination and immunoprecipitations were conducted as previously described (11). In some experiments, cells were lysed into 1% Brij 96 (Sigma Chemical Co., St. Louis, MO) instead of Nonidet P-40. Pulse-chase experiments on 6-h PMA-activated II-23 cells were performed by labeling the cells at 10^7 cells/ml in Met/Cys-free RPMI with 10% dialyzed FCS, 10 mM HEPES, glutamine, and 100 uCi/ml [^{35}S]TranSlabel (Dupont NEN, Boston, MA) for 6 min. Unlabeled methionine and cysteine were added to 0.1 mM each and aliquots of 10^7 cells were removed at various times, placed on ice, and subjected to immunoprecipitation analyses. The intensity of either ^{35}S- or ^{125}I-radiolabeled bands was quantitated by using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Table I. *Properties of anti-LT mAbs*

mAb	Isotype	Neutralization of Soluble LT-α[a]	FACS Staining Activated II23
Anti-LT-α mAbs			
9B9	IgG1	10	Bright
NC2	IgG2a	12	Bright
AG9	IgG1	15	Bright
FF2	IgG2a	>10,000	Bright
AA6	IgG1	1,500	Bright
GC4	IgG1	6,000	Bright
AH6	IgG2a	13	Dull
DH1	IgG2a	10	Dull
CH12	IgG2a	10	Dull
FE2	IgG2a	13	Dull
BF7	IgG1	100	Dull
BMS105	IgG2b	nd	None
Anti-LT-β mAbs			
B9	IgG1	>10,000	Bright
B27	IgG1	>10,000	Bright
C37	IgG1	>10,000	Bright
A3	IgG1	nd	None

[a] Concentration in ng/ml required to block 50% of the anti-growth activity of 10 ng/ml rh-LT-α (CHO derived) in a standard L929 assay. Similar results were obtained with either a 1-day cytolytic assay or 3-day antiproliferative assays. 9B9 and BMS105 are commercial mAbs from Boehringer Mannheim and Biosource, respectively. nd, not determined.

Biologic assays

TNF/LT activity was measured in either 1-day cytotoxicity or 3-day antiproliferative assays as previously described (17). In mAb neutralization studies, various amounts of mAb were premixed for 30 min with 1 ng of recombinant CHO cell-derived LT-α in a volume of 60 μl. Cells were then added to bring the volume to 0.1 ml.

Results

Anti-LT-α mAbs fall into two groups

A panel of anti-LT-α mAbs was prepared and characterized by their ability to stain activated II-23 cells, to neutralize rhLT-α in a standard L929 assay, and to immunoprecipitate the surface LT complex. These analyses are summarized in Table I and show that the mAbs can be grouped into two categories. It is possible that some clones within these groups arose from the same precursor cell. One group stains PMA-activated II-23 cells brightly whereas the other group stains poorly (Fig. 1). Earlier work indicated that the bulk of the surface LT-β could be cross-linked into a dimer, leading to the hypothesis that the major surface form had at least a LT-α1/β2 stoichiometry (9). These data suggest that the FACS dull group of mAbs recognizes only the minor LT-α2/β1 complex whereas the FACS bright set can bind to any LT-α-containing complex.

Immunoprecipitation experiments using [^{35}S]methionine-labeled PMA-activated II-23 cells were conducted to determine whether different complexes were recognized by these groups of mAbs. Activated II-23 cells not only

certain degree of overlap seems to exist between the binding sites for either type of TNF on TNFR1.

TNFα is thought to play an important role in the pathogenesis of various human diseases including septic shock (33, 34). We and others have developed TNFR1 immunoadhesins that block TNFα *in vivo* and thus protect against mortality in animal models for septic shock (33, 35). The studies reported here provide a more definitive framework than available previously for identifying the sequences in TNFR1 that interact with TNFα and TNFβ and potentially will be useful in the design of more potent and specific molecules that block TNF *in vivo*.

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59 Production and purification of the chimetic TNF inhibitor

60 For production of inhibitor cells were incubated in serum free medium (90% F12:10% complete WRC 935) for 72 hours. Cells were then removed by centrifugation followed by filtration. The conditioned medium was then passed over a column of goat anti-mouse heavy chain IgG coupled to sepharose (Sigma Chemical Co.). The affinity resin was then washed with a solution containing 500 mM NaCl, 10 mM NaH.sub.2 pO.sub.4 (pH 7.2), and 1 mM EDTA. Bound protein was eluted with a solution containing 50 mM acetic acid (pH 2.4) and 150 mM NaCl. 500 .mu.l fractions were collected and neutralized by addition of 75 .mu.l of 1 M Tris (pH 8.0). Fractions were dot-blotted onto nitrocellulose and incubated with alkaline phosphatase conjugated goat anti-mouse IgG to permit detection and quantitation of the inhibitor.

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